PATENT ABSTRACTS OF JAPAN

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(54) METHOD FOR MANUFACTURING CONDROITIN SULFURIC ACIDS

(57)Abstract:
PROBLEM TO BE SOLVED: To provide a method for manufacturing a condroitin sulfuric acid using a raw material which is easily available and by means of comparatively easy treatments.
SOLUTION: Fish scales are uses as a raw material. They are subjected to a dissolving treatment, and than are subjected to a polypeptide removing treatment and a fractionation treatment, in this order, so as to obtain a condroitin sulfuric acid. For example, the scale dissolving treatment is carried out by allowing a protease to act on the scales in water. Next, a polypeptide formed as a by-product is removed from the solution thus formed. Then a condroitin sulfuric acid is obtained by fractional precipitation.

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CLAIMS

[Claim(s)]
[Claim 1] UROKO of fishes -- a raw material -- carrying out -- this -- solubilization processing, polypeptide clearance processing, and judgment processing -- one by one -- ****** -- the manufacture approach of the chondroitin sulfate characterized by things.
[Claim 2] The manufacture approach of the chondroitin sulfate according to claim 1 which acquires chondroitin sulfate according to fractional precipitation after removing a byproduction polypeptide from the solubilization liquid obtained by making a protease act on UROKO of fishes, solubilizing UROKO underwater, and then doing in this way.
[Claim 3] The manufacture approach of chondroitin sulfate according to claim 1 to 2 that chondroitin sulfate is chondroitin sulfate A and chondroitin sulfate C.

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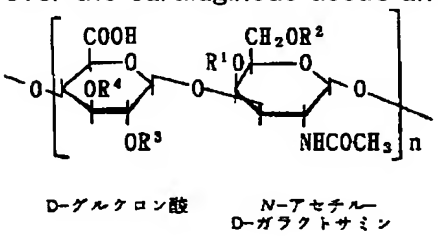
DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to the approach of manufacturing chondroitin sulfate useful as eye lotions or a remedy of neuralgia and the arthralgia especially chondroitin sulfate A, and chondroitin sulfate C, by using UROKO of fishes as a raw material.
[0002]
[Description of the Prior Art] Chondroitin sulfate is one sort of the typical glycosaminoglycan widely distributed over the cartilaginous tissue and connective tissue of an animal, and is a general formula [** 1].



It has the structure with which it came out and 40-100 main repeat units combined the disaccharide expressed. With the number and joint location of a sulfuric-acid radical chondroitin sulfate A (a chondroitin-4-sulfuric acid and R1=SO3H --) R2-R4=H and chondroitin sulfate C (a chondroitin-6-sulfuric acid --) R2=SO3H, R1 and R3, R4=H, and chondroitin sulfate D (R2 and R3=SO3H --) R1, R4=H, and chondroitin sulfate E (R1 and R2=SO3H --) It is divided into R3, R4=H, chondroitin sulfate K (R1, R4=SO4H, R2, R3=H), and chondroitin sulfate B (5-epimerization of many of dermatan sulfate and D-glucuronic acid is done, it serves as L-iduronic acid, and the R1 has become a sulfuric-acid radical).
[0003] the chondroitin sulfate A in these chondroitin sulfate -- mainly -- the notochord of a sturgeon, and the cartilagines nasi of a whale -- moreover, chondroitin sulfate C -- mainly -- a shark -- although the cartilage was manufactured as a raw material, respectively, since acquisition of a raw material needed complicated processing for a difficult top, mass production method was not completed and it did not escape becoming cost high.
[0004]
[Problem(s) to be Solved by the Invention] This invention is made using the raw material which is easy to come to hand for the purpose of manufacturing chondroitin sulfate by the easy processing in comparison.
[0005]
[Means for Solving the Problem] As a result of examining many things about the manufacture raw material of chondroitin sulfate, chondroitin sulfate A and chondroitin sulfate C are contained in UROKO of the fishes conventionally discarded while it had been unused, and this invention persons came to make this invention for the ability of these to be taken out comparatively easily based on a header and this knowledge.
[0006] The manufacture approach of the chondroitin sulfate characterized by things is offered, and this approach is underwater. namely, this invention -- UROKO of fishes -- a raw material -- carrying out -- this -- solubilization processing, polypeptide clearance processing, and judgment processing -- one by one -- ****** -- A protease is made to act on UROKO of fishes and UROKO is solubilized, and it consists of acquiring chondroitin sulfate according to fractional precipitation, after removing a byproduction polypeptide from the solubilization liquid obtained by next doing in this way.

[0007]
[Embodiment of the Invention] UROKO used in this invention approach may be which UROKO of freshwater fish and a saltwater fish. As UROKO of freshwater fish, UROKO, such as a carp, a crucian carp, a mass, and a goldfish, can be used, for example, and UROKO, such as Thailand, Suzuki, a salmon, a herring, and ETSU, can be used as UROKO of a saltwater fish, for example. Although it can be used only rinsing and removing dirt, if these UROKO is required, what was beforehand heated for 5 - 30 minutes at the temperature of 120-130 degrees C can also be used for it.
[0008] The solubilization processing in this invention approach adds and homogenizes the thin water solution of calcium acetate, and the water solution preferably buffered with the tris hydrochloric acid to UROKO of a raw material, is used as suspension, and is performed by making a protease add and react to this. This reaction is usually completed in the temperature of 30-40 degrees C in 5 - 40 hours. By this reaction, the protein part combined with chondroitin sulfate in UROKO dissociates, the byproduction of the polypeptide is carried out, with chondroitin sulfate, it dissolves in water and this becomes a water solution.
[0009] Thus, the obtained water solution is ** given to polypeptide clearance processing after filtration or centrifugal separation subsequently removes insoluble matter. This is performed by contacting the water solution except the above-mentioned insoluble matter on cation mold ion exchange resin. Under the present circumstances, it can precede contacting cation mold ion exchange resin, a water solution can be condensed if needed, and it can also dialyze to distilled water.
[0010] Thus, although the water solution containing chondroitin sulfate is obtained, in order to classify the object product from this water solution, by adding ethyl alcohol and raising concentration gradually into this water solution, chondroitin sulfate is settled and uptake is carried out for every fraction.
[0011] Thus, the chondroitin sulfate obtained from each fraction was identified by hydrolyzing with a digestive enzyme and detecting the saccharide to generate, making it contrast with the preparation of the origin which should be trusted. It hydrolyzes each sample using a chondroitinase, and this identification develops the obtained liquid by the thin film chromatography (it omits Following TLC), and the generated partial saturation disaccharide is made to color using a diphenylamine reagent, and it is performed by checking whether it is in agreement with the band of a preparation processed similarly. Thus, it was checked that the products of this invention approach are chondroitin sulfate A and chondroitin sulfate C.
[0012]
[Example] Next, an example explains this invention to a detail further.
[0013] solubilization of example 1 (1) UROKO -- a carp -- after rinsing UROKO 60g well, it heat-treated for 20 minutes at 120 degrees C. subsequently, UROKO heat-treated to 300ml (pH7.8) of 0.05M-tris hydrochloric-acid buffer solutions containing 0.02M-calcium acetate, and AKUCHINAZE E(Kaken Pharmaceutical make)300mg -- in addition, it homogenized with the homogenizer and suspension was prepared. Next, this suspension was made to react for three days at 37 degrees C, and UROKO was solubilized thoroughly. Thus, after having heated the obtained reaction mixture for 5 minutes at 100 degrees C, having carried out deactivation of AKUCHINAZE E, carrying out at-long-intervals alignment separation for 40 minutes and removing an insoluble element (20,000rpm), centrifugal supernatant liquid was condensed and one nights of concentrates were dialyzed to distilled water. It freeze-dried, after removing the polypeptide which separated at the above-mentioned reaction through the liquid which carried out dialysis processing in Dowex50-x8 (H+ mold) column (50x50mm) and neutralizing effluent by the sodium hydroxide. Thus, 330mg of freeze-drying objects was obtained.
[0014] (2) After dissolving 330mg of freeze-drying objects obtained by the fractional precipitation (1) of a meltable ghost in 50ml of 0.5M-sodium acetate, the acetic acid adjusted to pH4.5, ethanol was poured into this, by raising ethanol concentration gradually, chondroitin sulfate was settled and uptake was carried out as a fraction for every ethanol concentration. After adding 2 N-HCl of an amount (w/v) to each obtained fraction 1,000 times and hydrolyzing in 100 degrees C for 20 hours, each sugar composition was analyzed using the DX-500 sugar analysis apparatus (product made from die ONEKUSU). The result is shown in a table 1.
[0015]
[A table 1]

エタノール画分（%）	沈殿物収量（mg）	糖組成（μg／mg）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～40	5.0	11.15	43.54	13.38	47.46	18.23	2.69	0.00
40～50	49.0	11.67	28.23	129.41	157.37	29.62	1.18	0.00
50～60	4.0	14.01	33.75	32.50	64.74	27.76	3.49	0.00
60～70	6.0	17.50	69.02	6.79	60.09	35.09	41.52	12.22
70～	70.0	3.43	20.47	0.00	7.33	13.49	39.53	15.63

[0016] In addition, the code of the sugar in a table has following semantics.
Fuc : fructose Gal : Galactose GlcUA : Glucuronic-acid GalNAc:N-acetyl-galactosamine GlcNAc: N-acetylglucosamine NeuAc : N-acetylneuraminic acid NeuGc : N-glycolyl neuraminic acid [0017] As shown in this table, other glucuronic acid and N-acetyl galactosamine of a lot of than a fraction are contained in an ethanol fraction 40 to 50%, and the mole ratio of GlcUA and GalNAc is about 1:1. Moreover, the iduronic acid which is the constituent of chondroitin sulfate B was detected from neither of the fractions.
[0018] (3) An identification trial Every [of a chondroitin-sulfate-C preparation / preparation / which were obtained by (2) / 40 - 50% ethanol fraction 75microg and the chondroitin-sulfate-A preparation [it is written as the Seikagaku make speed signal generator grade, and Following ChsA] / [it is written as the Seikagaku make, speed signal generator grade, and Following ChsC] / 50micro / g] It dissolved in 100micro (pH5.0) of 0.025M sodium acetate buffer solutions I, respectively, and chondroitinase ASURO II[Seikagaku0.02] unit was made to melt and react to the 2micro of the same buffer solutions I as the above at 37 degrees C in addition for 20 hours. Subsequently, ethanol 300microl was added to reaction mixture, deactivation of the enzyme was carried out, and centrifugal separation removed insoluble matter.
[0019] It analyzed after condensing the obtained centrifugal supernatant liquid using the silica gel 60TLC plate (Merck Co. make). This result is shown in drawing 1 . As a developing solution of TLC in this case, 1-butanol / acetic acid / distilled water (capacity 2/1/1) was used, and the diphenylamine reagent was used for coloring of the partial saturation disaccharide generated by the reaction of chondroitinase ASURO II. Two partial saturation disaccharide bands were detected from the ethanol fraction 40 to 50%, and those mobility was in agreement with the band of two partial saturation disaccharides generated from ChsA and ChsC which were used as a preparation, respectively so that this drawing 1 might show.
[0020] the carp in example 2 example 1 (1) -- red sea bream UROKO 142g was used instead of UROKO 60g, it processed like the example 1, and 95mg of ethanol precipitate was obtained. The sugar composition for every ethanol fraction of this thing was analyzed like the example 1 (2). The result is shown in a table 2.
[0021]
[A table 2]

エタノール画分（%）	沈殿物収量（mg）	糖組成（μg／mg）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～40	6.1	5.30	167.44	26.53	181.04	29.13	23.07	0.00
40～50	23.3	2.40	102.91	197.65	264.73	83.68	11.25	7.85
50～60	11.7	0.44	53.58	25.50	57.11	33.75	58.51	55.34
60～70	5.2	4.10	4.77	6.66	93.67	0.00	99.41	29.32
70～	48.7	9.34	7.00	0.00	226.40	0.00	153.95	0.00

[0022] the carp in example 3 example 1 (1) -- ETSUUROKO 26g was used instead of UROKO 60g, it processed like the example 1, and 20.8mg of ethanol precipitate was obtained. The sugar composition for every ethanol fraction of this thing was analyzed like the example 1 (2). The result is shown in a table 3.
[0023]
[A table 3]

エタノール画分（%）	沈殿物収量（mg）	糖組成（μg／mg）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～50	2.4	19.40	22.13	87.23	94.63	0.00	24.49	0.00
50～60	3.4	18.03	6.74	2.73	82.91	0.00	44.60	0.00
60～70	2.6	23.07	6.75	3.00	65.98	0.00	23.46	9.72
70～	12.4	12.93	8.40	1.55	138.72	0.00	113.39	25.76

[0024] the carp in example 4 example 1 (1) -- Lateolabrax UROKO 23g was used instead of UROKO 60g, it processed like the example 1, and 11.0mg of ethanol precipitate was obtained. The sugar composition for every ethanol fraction of this thing was analyzed like the example 1 (2). The result is shown in a table 4.

[0025]

[A table 4]

エタノール画分（%）	沈殿物収量（mg）	糖組成（μg／mg）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～50	1.4	4.91	11.01	67.92	82.36	0.00	11.57	0.00
50～60	1.7	1.93	131.13	2.63	14.25	0.00	15.03	0.00
60～70	1.2	6.19	8.14	0.00	27.09	0.00	18.95	0.00
70～	6.7	7.62	4.66	0.00	175.68	0.00	112.81	12.59

[0026] Although chondroitin sulfate A and chondroitin sulfate C are contained in the protease processing object of fishes UROKO as shown in a table 2 thru/or 4, chondroitin sulfate B is not contained.

[0027]

[Effect of the Invention] According to this invention, the high chondroitin sulfate A and the chondroitin sulfate C of utility value can be manufactured by easy actuation as a remedy by using as a raw material UROKO of the fishes which are one sort of fishery trash.

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TECHNICAL FIELD

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PRIOR ART

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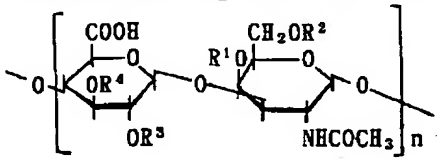

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EFFECT OF THE INVENTION

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TECHNICAL PROBLEM

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MEANS

[Means for Solving the Problem] As a result of examining many things about the manufacture raw material of chondroitin sulfate, chondroitin sulfate A and chondroitin sulfate C are contained in UROKO of the fishes conventionally discarded while it had been unused, and this invention persons came to make this invention for the ability of these to be taken out comparatively easily based on a header and this knowledge.
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[0009] Thus, the obtained water solution is ** given to polypeptide clearance processing after filtration or centrifugal separation subsequently removes insoluble matter. This is performed by contacting the water solution except the above-mentioned insoluble matter on cation mold ion exchange resin. Under the present circumstances, it can precede contacting cation mold ion exchange resin, a water solution can be condensed if needed, and it can also dialyze to distilled water.
[0010] Thus, although the water solution containing chondroitin sulfate is obtained, in order to classify the object product from this water solution, by adding ethyl alcohol and raising concentration gradually into this water solution, chondroitin sulfate is settled and uptake is carried out for every fraction.
[0011] Thus, the chondroitin sulfate obtained from each fraction was identified by hydrolyzing with a digestive enzyme and detecting the saccharide to generate, making it contrast with the preparation of the origin which should be trusted. It hydrolyzes each sample using a chondroitinase, and this identification develops the obtained liquid by the thin film chromatography (it omits Following TLC), and the generated partial saturation disaccharide is made to color using a diphenylamine reagent, and it is performed by checking whether it is in agreement with the band of a preparation processed similarly. Thus, it was checked that the products of this invention approach are chondroitin sulfate A and chondroitin sulfate C.

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EXAMPLE

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[0014] (2) After dissolving 330mg of freeze-drying objects obtained by the fractional precipitation (1) of a meltable ghost in 50ml of 0.5M-sodium acetate, the acetic acid adjusted to pH4.5, ethanol was poured into this, by raising ethanol concentration gradually, chondroitin sulfate was settled and uptake was carried out as a fraction for every ethanol concentration. After adding 2 N-HCl of an amount (w/v) to each obtained fraction 1,000 times and hydrolyzing in 100 degrees C for 20 hours, each sugar composition was analyzed using the DX-500 sugar analysis apparatus (product made from die ONEKUSU). The result is shown in a table 1.
[0015]
[A table 1]

エタノール画分（%）	沈殿物収量（mg）	糖組成（μg／mg）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～40	5.0	11.15	43.54	13.38	47.46	18.23	2.69	0.00
40～50	49.0	11.67	28.23	129.41	157.37	29.62	1.18	0.00
50～60	4.0	14.01	33.75	32.50	64.74	27.76	3.49	0.00
60～70	6.0	17.50	69.02	6.79	60.09	35.09	41.52	12.22
70～	70.0	3.43	20.47	0.00	7.33	13.49	39.53	15.63

[0016] In addition, the code of the sugar in a table has following semantics.
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[0018] (3) An identification trial Every [of a chondroitin-sulfate-C preparation / preparation / which were obtained by (2) / 40 - 50% ethanol fraction 75microg and the chondroitin-sulfate-A preparation [it is written as the Seikagaku make speed signal generator grade, and Following ChsA] / [it is written as the Seikagaku make, speed signal generator grade, and Following ChsC] / 50micro / g] It dissolved in 100micro (pH5.0) of 0.025M sodium acetate buffer solutions I, respectively, and chondroitinase ASURO II[Seikagaku0.02] unit was made to melt and react to the 2micro of the same buffer solutions I as the above at 37 degrees C in addition for 20 hours. Subsequently, ethanol 300microl was added to reaction mixture, deactivation of the enzyme was carried

out, and centrifugal separation removed insoluble matter.

[0019] It analyzed after condensing the obtained centrifugal supernatant liquid using the silica gel 60TLC plate (Merck Co. make). This result is shown in drawing 1 . As a developing solution of TLC in this case, 1-butanol / acetic acid / distilled water (capacity 2/1/1) was used, and the diphenylamine reagent was used for coloring of the partial saturation disaccharide generated by the reaction of chondroitinase ASURO II. Two partial saturation disaccharide bands were detected from the ethanol fraction 40 to 50%, and those mobility was in agreement with the band of two partial saturation disaccharides generated from ChsA and ChsC which were used as a preparation, respectively so that this drawing 1 might show.

[0020] the carp in example 2 example 1 (1) — red sea bream UROKO 142g was used instead of UROKO 60g, it processed like the example 1, and 95mg of ethanol precipitate was obtained. The sugar composition for every ethanol fraction of this thing was analyzed like the example 1 (2). The result is shown in a table 2.

[0021]

[A table 2]

エタノール画分（%）	沈殿物収量（mg）	糖組成（μg／mg）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～40	6.1	5.30	167.44	26.53	181.04	29.13	23.07	0.00
40～50	23.3	2.40	102.91	197.65	264.73	83.68	11.25	7.85
50～60	11.7	0.44	53.58	25.50	57.11	33.75	58.51	55.34
60～70	5.2	4.10	4.77	6.66	93.67	0.00	99.41	29.32
70～	48.7	9.34	7.00	0.00	226.40	0.00	153.95	0.00

[0022] the carp in example 3 example 1 (1) — ETSUUROKO 26g was used instead of UROKO 60g, it processed like the example 1, and 20.8mg of ethanol precipitate was obtained. The sugar composition for every ethanol fraction of this thing was analyzed like the example 1 (2). The result is shown in a table 3.

[0023]

[A table 3]

エタノール画分（%）	沈殿物収量（mg）	糖組成（μg／mg）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～50	2.4	19.40	22.13	87.23	94.63	0.00	24.49	0.00
50～60	3.4	18.03	6.74	2.73	82.91	0.00	44.60	0.00
60～70	2.6	23.07	6.75	3.00	65.98	0.00	23.46	9.72
70～	12.4	12.93	8.40	1.55	138.72	0.00	113.39	25.76

[0024] the carp in example 4 example 1 (1) — Lateolabrax UROKO 23g was used instead of UROKO 60g, it processed like the example 1, and 11.0mg of ethanol precipitate was obtained. The sugar composition for every ethanol fraction of this thing was analyzed like the example 1 (2). The result is shown in a table 4.

[0025]

[A table 4]

エタノール画分（%）	沈殿物収量（mg）	糖組成（μg／mg）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～50	1.4	4.91	11.01	67.92	82.36	0.00	11.57	0.00
50～60	1.7	1.93	131.13	2.63	14.25	0.00	15.03	0.00
60～70	1.2	6.19	8.14	0.00	27.09	0.00	18.95	0.00
70～	6.7	7.62	4.66	0.00	175.68	0.00	112.81	12.59

[0026] Although chondroitin sulfate A and chondroitin sulfate C are contained in the protease processing object of fishes UROKO as shown in a table 2 thru/or 4, chondroitin sulfate B is not contained.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] The pattern in which the result of TLC analysis of the ethanol precipitate obtained in the example 1 and each preparation is shown.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
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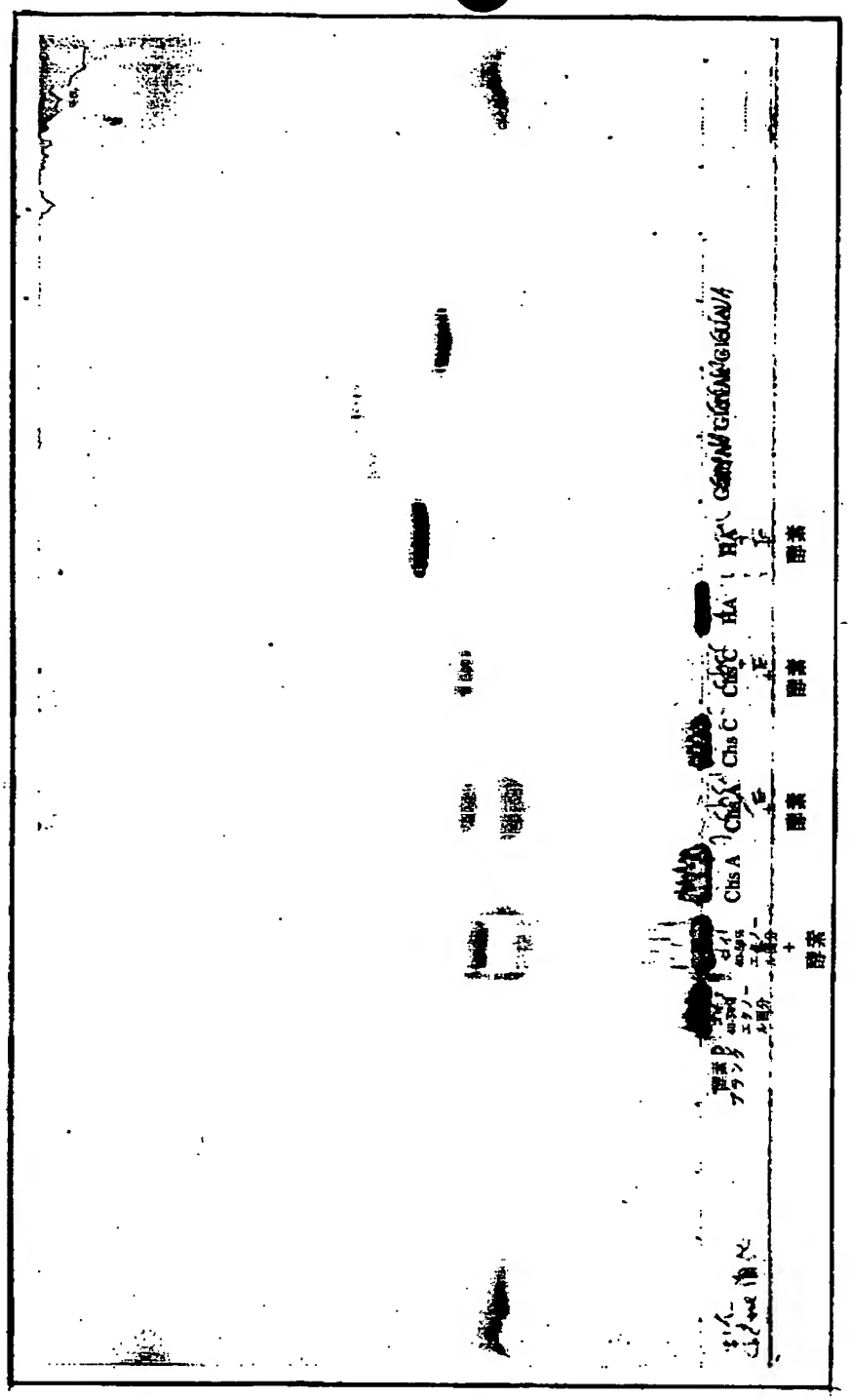
3.In the drawings, any words are not translated.

DRAWINGS

[Drawing 1]



[Translation done.]

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最終頁に続く

(54)【発明の名称】　コンドロイチン硫酸類の製造方法

(57)【要約】
【課題】　入手しやすい原料を用い、比較的に簡単な処理でコンドロイチン硫酸類を製造する。
【解決手段】　魚類のウロコを原料とし、これに可溶化処理、ポリペプチド除去処理及び分別処理を順次施こす。

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【特許請求の範囲】

【請求項１】　魚類のウロコを原料とし、これに可溶化処理、ポリペプチド除去処理及び分別処理を順次施こすことを特徴とするコンドロイチン硫酸類の製造方法。

【請求項２】　水中で、魚類のウロコにプロテアーゼを作用させて、ウロコを可溶化し、次にこのようにして得られた可溶化液から副生ポリペプチドを除去したのち、分別沈殿によりコンドロイチン硫酸類を取得する請求項１記載のコンドロイチン硫酸類の製造方法。

【請求項３】　コンドロイチン硫酸類がコンドロイチン硫酸Ａ及びコンドロイチン硫酸Ｃである請求項１ないし２のいずれかに記載のコンドロイチン硫酸類の製造方法。

【発明の詳細な説明】

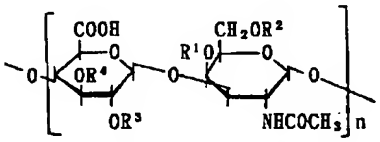
【０００１】

【発明の属する技術分野】本発明は、魚類のウロコを原料として、点眼液や神経痛、関節痛の治療薬として有用なコンドロイチン硫酸類、特にコンドロイチン硫酸Ａ及びコンドロイチン硫酸Ｃを製造する方法に関するものである。

【０００２】

【従来の技術】コンドロイチン硫酸は、動物の軟骨組織や結合組織に広く分布している代表的なグリコサミノグリカンの１種であって、一般式

【化１】



D-グルクロン酸　　N-アセチル-D-ガラクトサミン

で表わされる二糖を主要繰り返し単位４０～１００個が結合した構造を有し、硫酸基の数及び結合位置によって、コンドロイチン硫酸Ａ（コンドロイチン－４－硫酸、R^1＝SO_3Ｈ、R^2～R^4＝Ｈ）、コンドロイチン硫酸Ｃ（コンドロイチン－６－硫酸、R^2＝SO_3Ｈ、R^1，R^3，R^4＝Ｈ）、コンドロイチン硫酸Ｄ（R^2，R^3＝SO_3Ｈ、R^1，R^4＝Ｈ）、コンドロイチン硫酸Ｅ（R^1，R^2＝SO_3Ｈ、R^3，R^4＝Ｈ）、コンドロイチン硫酸Ｋ（R^1，R^4＝SO_4Ｈ、R^2，R^3＝Ｈ）、コンドロイチン硫酸Ｂ（デルマタン硫酸、Ｄ－グルクロン酸の多くが５－エピマー化されてＬ－イズロン酸となり、そのR^1が硫酸基になっている）に分けられている。

【０００３】これらのコンドロイチン硫酸類の中のコンドロイチン硫酸Ａは、主としてチョウザメの脊索、クジラの鼻軟骨を、またコンドロイチン硫酸Ｃは、主としてサメ軟骨をそれぞれ原料として製造されているが、原料の入手が困難な上に、煩雑な処理を必要とするため、大

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量生産ができず、コスト高になるのを免れなかった。

【０００４】

【発明が解決しようとする課題】本発明は、入手しやすい原料を用い、比較的に簡単な処理でコンドロイチン硫酸類を製造することを目的としてなされたものである。

【０００５】

【課題を解決するための手段】本発明者らは、コンドロイチン硫酸の製造原料について種々検討した結果、従来未利用のまま廃棄されていた魚類のウロコの中に、コンドロイチン硫酸Ａ及びコンドロイチン硫酸Ｃが含まれ、これらは比較的容易に取り出すことができることを見出し、この知見に基づいて本発明をなすに至った。

【０００６】すなわち、本発明は、魚類のウロコを原料とし、これに可溶化処理、ポリペプチド除去処理及び分別処理を順次施こすことを特徴とするコンドロイチン硫酸類の製造方法を提供するものであり、この方法は、例えば水中で、魚類のウロコにプロテアーゼを作用させて、ウロコを可溶化し、次にこのようにして得られた可溶化液から副生ポリペプチドを除去したのち、分別沈殿によりコンドロイチン硫酸類を取得することからなっている。

【０００７】

【発明の実施の形態】本発明方法において用いるウロコは、淡水魚、海水魚のいずれのウロコであってもよい。淡水魚のウロコとしては、例えばコイ、フナ、マス、キンギョなどのウロコを用いることができるし、海水魚のウロコとしては、例えばタイ、スズキ、サケ、ニシン、エツなどのウロコを用いることができる。これらのウロコは、単に水洗して汚れを除いたままで使用することができるが、必要ならば、あらかじめ１２０～１３０℃の温度で５～３０分間加熱したものを用いることもできる。

【０００８】本発明方法における可溶化処理は、原料のウロコに酢酸カルシウムの希薄水溶液、好ましくはトリス塩酸で緩衝された水溶液を加え、ホモジナイズして懸濁液とし、これにプロテアーゼを加えて反応させることによって行われる。この反応は、通常３０～４０℃の温度において、５～４０時間で完了する。この反応により、ウロコ中でコンドロイチン硫酸類と結合していたタンパク質部分が分離してポリペプチドを副生し、これはコンドロイチン硫酸類とともに水に溶解して水溶液になる。

【０００９】このようにして得た水溶液は、次いで不溶分をろ過又は遠心分離により除去したのち、ポリペプチド除去処理に付する。これは、上記の不溶分を除いた水溶液をカチオン型イオン交換樹脂と接触させることにより行われる。この際、カチオン型イオン交換樹脂と接触するに先立って、必要に応じ水溶液を濃縮し、蒸留水に対して透析することもできる。

【００１０】このようにして、コンドロイチン硫酸類を

含む水溶液が得られるが、この水溶液から目的生成物を分別するには、この水溶液中にエチルアルコールを加え、濃度を段階的に上げることによって、コンドロイチン硫酸類を沈殿させ、各画分ごとに捕集する。

【００１１】このようにして、各画分から得られたコンドロイチン硫酸類は、信頼すべき由来の標品と対比させながら、消化酵素により加水分解し、生成する糖類を検出することにより同定された。この同定は、例えばコンドロイチナーゼを用いて各試料を加水分解し、得られた液を薄膜クロマトグラフィ（以下ＴＬＣと略す）により展開し、生成した不飽和二糖をジフェニルアミン試薬を用いて発色させ、同様に処理した標品のバンドと一致するか否かを確認することによって行われる。このようにして、本発明方法の生成物は、コンドロイチン硫酸Ａ及びコンドロイチン硫酸Ｃであることが確認された。

【００１２】

【実施例】次に実施例により本発明をさらに詳細に説明する。

【００１３】実施例１

（１）ウロコの可溶化

コイウロコ６０ｇをよく水洗したのち、１２０℃で２０分間加熱処理した。次いで、０．０２Ｍ－酢酸カルシウムを含む０．０５Ｍ－トリス塩酸緩衝液（ｐＨ７．８）３００ｍｌに加熱処理したウロコとアクチナーゼＥ（科研製薬製）３００ｍｇを加えて、ホモジナイザーにより＊均質化し、懸濁液を調製した。次にこの懸濁液を３７℃で３日間反応させ、ウロコを完全に可溶化した。このようにして得た反応液を１００℃で５分間加熱してアクチナーゼＥを失活させ、４０分間遠心分離して（２０，０００ｒｐｍ）不溶成分を除去したのち、遠心上清を濃縮し、濃縮物を蒸留水に対して１夜透析した。透析処理した液を、Ｄｏｗｅｘ５０－ｘ８（H^+ 型）カラム（５０×５０ｍｍ）に通して上記の反応で遊離したポリペプチドを除去し、流出液を水酸化ナトリウムで中和したのち、凍結乾燥した。このようにして凍結乾燥物３３０ｍｇを得た。

【００１４】（２）可溶化物の分別沈殿

（１）で得た凍結乾燥物３３０ｍｇを０．５Ｍ－酢酸ナトリウム５０ｍｌに溶解したのち、酢酸によりｐＨ４．５に調整し、この中にエタノールを注加し、エタノール濃度を段階的に上げることによってコンドロイチン硫酸を沈殿させ、エタノール濃度ごとの画分として捕集した。得られた各画分に１，０００倍量（ｗ／ｖ）の２Ｎ－ＨＣｌを加え、１００℃において２０時間加水分解したのち、ＤＸ－５００糖分析装置（ダイオネクス社製）を用いてそれぞれの糖組成を分析した。その結果を表１に示す。

【００１５】

【表１】

エタノール画分（%）	沈殿物収量（ｍｇ）	糖組成（μｇ／ｍｇ）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～40	5.0	11.15	43.54	13.38	47.46	18.23	2.69	0.00
40～50	49.0	11.67	28.23	129.41	157.37	29.62	1.18	0.00
50～60	4.0	14.01	33.75	32.50	64.74	27.76	3.49	0.00
60～70	6.0	17.50	69.02	6.79	60.09	35.09	41.52	12.22
70～	70.0	3.43	20.47	0.00	7.33	13.49	39.53	15.63

【００１６】なお、表中の糖の略号は以下の意味をもつ。

Ｆｕｃ　：フルクトース
Ｇａｌ　：ガラクトース
ＧｌｃＵＡ　：グルクロン酸
ＧａｌＮＡｃ：Ｎ－アセチルガラクトサミン
ＧｌｃＮＡｃ：Ｎ－アセチルグルコサミン
ＮｅｕＡｃ　：Ｎ－アセチルノイラミン酸
ＮｅｕＧｃ　：Ｎ－グリコリルノイラミン酸

【００１７】この表から分るように、４０～５０%エタノール画分には他の画分よりも多量のグルクロン酸及びＮ－アセチルガラクトサミンが含まれ、またＧｌｃＵＡとＧａｌＮＡｃとのモル比はほぼ１：１である。また、いずれの画分からもコンドロイチン硫酸Ｂの構成成分であるイズロン酸は検出されなかった。

【００１８】（３）同定試験

（２）で得た４０～５０%エタノール画分７５μｇ及びコンドロイチン硫酸Ａ標品［生化学工業（株）製，ＳＳＧグレード，以下ＣｈｓＡと略記する］とコンドロイチン硫酸Ｃ標品［生化学工業（株）製，ＳＳＧグレード，以下ＣｈｓＣと略記する］の５０μｇずつを、それぞれ０．０２５Ｍ酢酸ナトリウム緩衝液（ｐＨ５．０）１００μｌに溶解し、コンドロイチナーゼ・アースロＩＩ［生化学工業（株）製］０．０２単位を前記と同じ緩衝液２μｌに溶かして加え、３７℃で２０時間反応させた。次いで反応液にエタノール３００μｌを加えて酵素を失活させ、遠心分離により不溶分を除去した。

【００１９】得られた遠心上清を濃縮後、シリカゲル６０ＴＬＣプレート（メルク社製）を用いて分析した。この結果を図１に示す。この際のＴＬＣの展開液としては１－ブタノール／酢酸／蒸留水（容量２／１／１）を使用し、コンドロイチナーゼ・アースロＩＩの反応によって生成する不飽和二糖の発色にはジフェニルアミン試薬を用いた。この図１から分るように、４０～５０%エタ

ノール画分から２個の不飽和二糖バンドが検出され、それらの移動度は標品として用いたＣｈｓＡ及びＣｈｓＣから生成する２個の不飽和二糖のバンドとそれぞれ一致した。

【００２０】実施例２

実施例１（１）におけるコイウロコ６０ｇの代りにマダ＊イウロコ１４２ｇを用い、実施例１と同様に処理して、エタノール沈殿物９５ｍｇを得た。このものの各エタノール画分ごとの糖組成を実施例１（２）と同様にして分析した。その結果を表２に示す。

【００２１】

【表２】

エタノール画分（%）	沈殿物収量（ｍｇ）	糖組成（μｇ／ｍｇ）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～40	6.1	5.30	167.44	26.53	181.04	29.13	23.07	0.00
40～50	23.3	2.40	102.91	197.65	264.73	83.68	11.25	7.85
50～60	11.7	0.44	53.58	25.50	57.11	33.75	58.51	55.34
60～70	5.2	4.10	4.77	6.66	93.67	0.00	99.41	29.32
70～	48.7	9.34	7.00	0.00	226.40	0.00	153.95	0.00

【００２２】実施例３

実施例１（１）におけるコイウロコ６０ｇの代りにエツウロコ２６ｇを用い、実施例１と同様に処理して、エタノール沈殿物２０．８ｍｇを得た。このものの各エタノ※ール画分ごとの糖組成を実施例１（２）と同様にして分析した。その結果を表３に示す。

【００２３】

【表３】

エタノール画分（%）	沈殿物収量（ｍｇ）	糖組成（μｇ／ｍｇ）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～50	2.4	19.40	22.13	87.23	94.63	0.00	24.49	0.00
50～60	3.4	18.03	6.74	2.73	82.91	0.00	44.60	0.00
60～70	2.6	23.07	6.75	3.00	65.98	0.00	23.46	9.72
70～	12.4	12.93	8.40	1.55	138.72	0.00	113.39	25.76

【００２４】実施例４

実施例１（１）におけるコイウロコ６０ｇの代りにスズキウロコ２３ｇを用い、実施例１と同様に処理して、エタノール沈殿物１１．０ｍｇを得た。このものの各エタ★ノール画分ごとの糖組成を実施例１（２）と同様にして分析した。その結果を表４に示す。

【００２５】

【表４】

エタノール画分（%）	沈殿物収量（ｍｇ）	糖組成（μｇ／ｍｇ）						
		Fuc	Gal	GlcUA	GalNAc	GlcNAc	NeuAc	NeuGc
0～50	1.4	4.91	11.01	67.92	82.36	0.00	11.57	0.00
50～60	1.7	1.93	131.13	2.63	14.25	0.00	15.03	0.00
60～70	1.2	6.19	8.14	0.00	27.09	0.00	18.95	0.00
70～	6.7	7.62	4.66	0.00	175.68	0.00	112.81	12.59

【００２６】表２ないし４から分るように、魚類ウロコのプロテアーゼ処理物中には、コンドロイチン硫酸Ａ及びコンドロイチン硫酸Ｃが含まれているが、コンドロイチン硫酸Ｂは含まれていない。

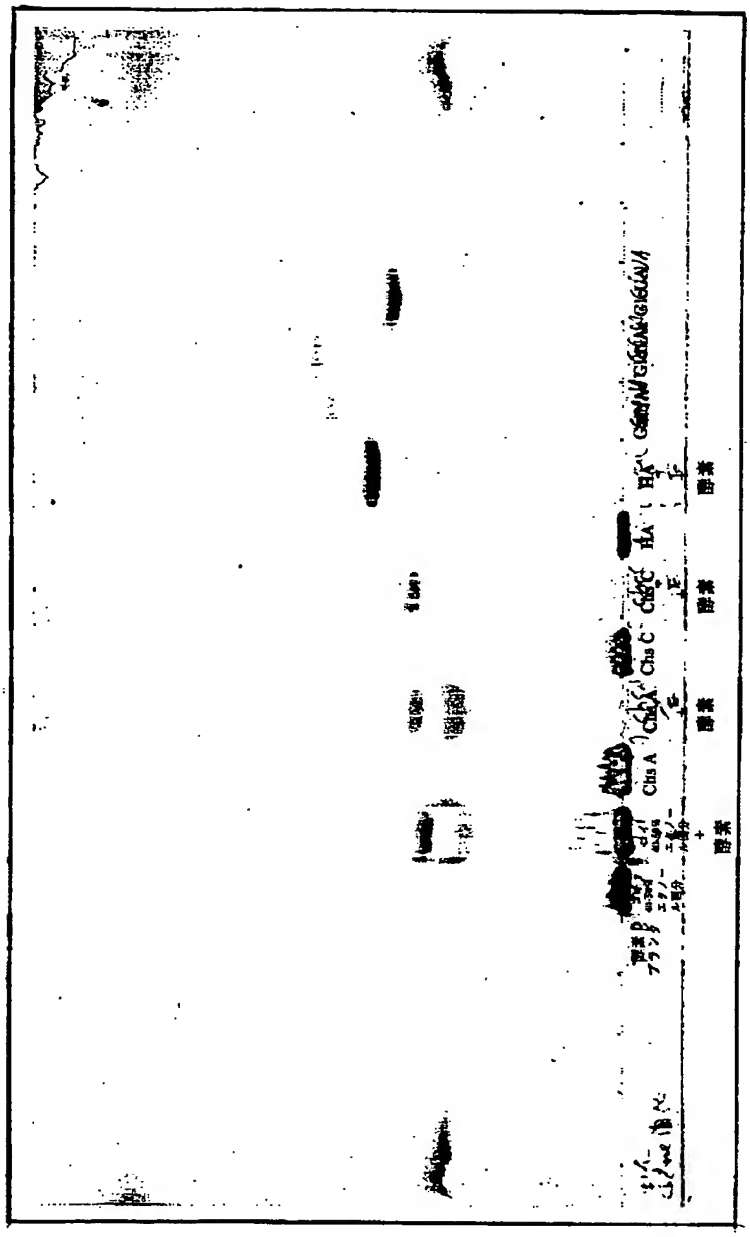
【００２７】

【発明の効果】本発明によると、漁業廃棄物の１種である魚類のウロコを原料として、医薬として利用価値の高いコンドロイチン硫酸Ａ及びコンドロイチン硫酸Ｃを簡単な操作で製造することができる。

【図面の簡単な説明】

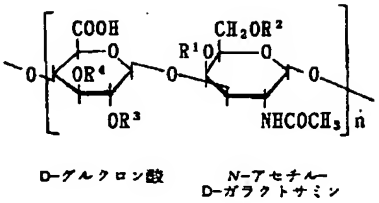
【図１】　実施例１で得たエタノール沈殿物と各標品のＴＬＣ分析の結果を示すパターン。

【図１】



【手続補正書】
【提出日】平成１２年５月１５日（２０００．５．１５）
【手続補正１】
【補正対象書類名】明細書
【補正対象項目名】０００２
【補正方法】変更
【補正内容】
【０００２】
【従来の技術】コンドロイチン硫酸は、動物の軟骨組織や結合組織に広く分布している代表的なグリコサミノグ

リカンの１種であって、一般式
【化１】



D-グルクロン酸　　N-アセチル-D-ガラクトサミン

で表わされる二糖を主要繰り返し単位４０～１００個が結合した構造を有し、硫酸基の数及び結合位置によって、コンドロイチン硫酸A（コンドロイチン－４－硫酸、R^1＝SO_3H、R^2～R^4＝H）、コンドロイチン硫酸C（コンドロイチン－６－硫酸、R^2＝SO_3H、R^1，R^3，R^4＝H）、コンドロイチン硫酸D（R^2，R^3＝SO_3H、R^1，R^4＝H）、コンドロイチン硫酸E（R^1，R^2＝SO_3H、R^3，R^4＝H）、コンドロイチン硫酸K（R^1，R^4＝SO_3H、＊R^2，R^3＝H）、コンドロイチン硫酸B（デルマタン硫酸、D－グルクロン酸の多くが５－エピマー化されてL－イズロン酸となり、そのR^1が硫酸基になっている）に分けられている。

【手続補正２】
【補正対象書類名】明細書
【補正対象項目名】００１６
【補正方法】変更
【補正内容】
【００１６】なお、表中の糖の略号は以下の意味をもつ。
Ｆｕｃ　　：フコース
Ｇａｌ　　：ガラクトース
ＧｌｃＵＡ　：グルクロン酸
ＧａｌＮＡｃ：Ｎ－アセチルガラクトサミン
ＧｌｃＮＡｃ：Ｎ－アセチルグルコサミン
ＮｅｕＡｃ　：Ｎ－アセチルノイラミン酸
ＮｅｕＧｃ　：Ｎ－グリコリルノイラミン酸

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